the immune fraction (Fig. 2). Each of the eight bands from fractionated immune hemolymph was cut from simultaneously run unstained gels, and the protein was eluted into a small volume of dilute salt solution (*9*) by ultrasonic mixing with a microprobe. After clarification by centrifugation, material obtained from each band was assayed for immobilizing effect in the standard test system. Activity was obtained in the C band present in immune hemolymph. The C band present in normal male hemolymph does not show this activity, nor do samples in any of the three proteins present only in the fraction from immunized hemolymph. The new proteins in the immune hemolymph fraction are thus evidently formed in response to portions of the ciliate that are not associated with the immobilizing response, whereas, like the gamma globulin response in mammals, the immune material is normally present as a preformed protein fraction in the circulating fluid of the organism and becomes altered in response to immunization.

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## Induction of Collagenolytic and Proteolytic Activities in Rat and Human Fibroblasts by Anti-Inflammatory Drugs

Abstract. *Confluent monolayers of either mouse or diploid human fibroblasts contained no measurable amounts of either collagenolytic (pH 5.5) or proteolytic (pH 7.5) activities. Within a few hours after exposure of these cells to anti-inflammatory drugs, significant amounts of these enzymatic activities were demonstrable extracellularly. These activities were profoundly decreased in cultures simultaneously treated with the anti-inflammatory drugs and cycloheximide or actinomycin D.*

Following the administration of corticosteroids, loss of collagen from the skin of rats is marked (*1*). A similar loss has been shown in man (*2*). In the rat, the biological half-life of insoluble cutaneous collagen is about 1 year, and the total loss of collagen in 24 hours is about 50 mg for a 200-g rat (*3*). During the day after administration of cortisol, indomethacin, and oxyphenylbutazone, but not salicylate, catabolism of collagen in the skin is more than ten times that occurring normally in the whole body (*4*). It seems unlikely that these drugs could effect such a loss by merely inhibiting the anabolism and not accelerating the catabolism of this fibrous protein.

Since collagen is located extracellularly, and since the administration of these anti-inflammatory drugs neither increases the number nor alters the kinds of cells found in the skin (*2*), catabolism of cutaneous collagen must proceed by the release of collagenolytic activity from normal skin cells into the extracellular compartment of the tissue. The skin contains enzymatic activities capable of digesting purified insoluble cutaneous collagen at *p*H 5.5 (*5*). Free collagenolytic activity and a proteolytic activity (*p*H 7.5) were found in the extracellular compartment of the skin from rats treated with steroids, neither was found in untreated control rats (*4*, *6*). Their appearance was always associated with a loss of collagen from the skin (*4*, *6*). The enzyme activities were not associated with the release of any of the well-known enzymes marking the labilization of lysosomes (*4*, *7*).

Finally, previous treatment of rats with such varied inhibitors of protein biosynthesis as puromycin [inhibiting messenger RNA (*8*)], cycloheximide [inhibiting peptide chain lengthening (*9*)], and actinomycin D [inhibiting messenger RNA synthesis (*10*)] inhibited the extracellular appearance of the collagenolytic and proteolytic activities and the loss of insoluble collagen from the skin (*11*). Previous treatment of cortisol-treated rats with 5,5-diphenylhydantoin also inhibited the appearance of collagenolytic activity and loss of insoluble collagen from the skin (*11*). Our results suggest that the induction of these enzymatic activities results from the drug-produced derepression of an operon within the mouse heteroploid or human diploid fibroblast in confluent monolayer cell culture.

Strain L fibroblasts were obtained (*12*) and grown in Eagle's essential medium containing 10 percent fetal calf serum in the usual fashion in 32-ounce pharmacy bottles until they reached confluency. The confluent monolayers of these cells (more than $20 \times 10^6$ cells per bottle) were allowed to stand for 5 days in medium to which had been added 12 $\mu$g of ascorbate per milliliter. The monolayer was then rinsed with sterile isotonic saline until no more protein could be determined (*13*) in the washings. These monolayers were then harvested, counted in a hemocytometer, and suspended in 1 ml of medium (without serum or ascorbic acid) per $4 \times 10^6$ cells. To some of these cell suspensions was added cortisol succinate, indomethacin, or oxyphenylbutazone (20 $\mu$g/ml). Some of these treated cultures also received simultaneously either cycloheximide or actinomycin D (5 $\mu$g/ml). After 0, 2, 3, 4, 5, and 6 hours, samples of medium and cells were collected together, homogenized in a Potter glass homogenizer, and assayed for proteolytic and collagenolytic activities.

Proteolytic activity was determined by incubation of denatured hemoglobin at *p*H 7.5 with 1 ml of homogenate for 16 hours at 36°C (*14*). The amount of protein soluble in trichloroacetic acid released under these conditions was expressed in micrograms of tyrosine equivalents per $4 \times 10^6$ cells (*4*, *14*).

Collagenolytic activity was determined by incubation of 1 ml of the homogenate with 10 mg of purified, native, insoluble rat-skin collagen at *p*H 5.5 for 16 hours at 24°C (*4*, *6*). The amount of peptide-bound hydroxyproline rendered soluble under these conditions was determined after hydrolysis in 4*N* HCl at 100°C for 8½ hours (*15*). The results were expressed in micrograms of hydroxyproline released per $4 \times 10^6$ cells.

The insoluble cutaneous collagen substrate was prepared as described previously (*4*–*6*) and contained less than 2 percent noncollagenous nitrogen.

Table 1. The effects of three inducing drugs (20 $\mu$g/ml) on the proteolytic activity of L strain fibroblasts in confluent monolayer (micrograms of trichloroacetic acid–soluble tyrosine equivalents per $4 \times 10^6$ cells) either alone or with cycloheximide (cyclo) or actinomycin D (actino) (5 $\mu$g/ml).

| Hours after drug | Oxyphenylbutazone | | | Cortisol | | | Indomethacin | | |
|---|---|---|---|---|---|---|---|---|---|
| | Alone | +Cyclo | +Actino | Alone | +Cyclo | +Actino | Alone | +Cyclo | +Actino |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | * 48 | * 7 | *0* | *10 | 0 | *0* | * 50 | *0* | *0* |
| 3 | *110 | *10* | * 5* | *68 | *10* | *0* | * 90 | *10* | *0* |
| 4 | *135 | *17* | *10* | *78 | *10* | *5* | *135 | *10* | *8* |
| 5 | *130 | * 6* | * 8* | *80 | * 7 | *0* | *128 | * 8* | *8* |
| 6 | *138 | *5* | * 7* | *80 | *6* | *0* | *125 | *5* | *0* |

* Significantly different from zero-hour control ($P < .05$); means in italics were significantly different from the appropriate "drug control" column.

Table 2. The effects of three inducing drugs (20 $\mu$g/ml) upon the collagenolytic activity of L strain fibroblasts in confluent monolayer (micrograms of soluble hydroxyproline per $4 \times 10^6$ cells) either alone or with cycloheximide (cyclo) or actinomycin D (actino) (5 $\mu$g/ml).

| Hours after drug | Oxyphenylbutazone | | | Cortisol | | | Indomethacin | | |
|---|---|---|---|---|---|---|---|---|---|
| | Alone | +Cyclo | +Actino | Alone | +Cyclo | +Actino | Alone | +Cyclo | +Actino |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | *28 | 0 | 0 | * 8 | *0* | *0* | *20 | *0* | *0* |
| 3 | *64 | *0* | *0* | *32 | *0* | *0* | *48 | *0* | *0* |
| 4 | *84 | *8* | *4* | *64 | *4* | *0* | *76 | *8* | *4* |
| 5 | *92 | *8* | *4* | *64 | *8* | *4* | *84 | *8* | *4* |
| 6 | *92 | *0* | *0* | *64 | *4* | *0* | *84 | *8* | *0* |

* Significantly different from zero-hour control ($P < .05$); means in italics were significantly different from the appropriate "drug control" column.

None of the hydroxyproline of this substrate could be solubilized with 1 mg of trypsin per milliliter at *p*H 5.5 or by buffer alone after overnight incubation at 24°C.

All determinations were done in duplicate, and each experiment was performed at least three times in triplicate culture bottles. All results were expressed as the mean value of at least three experiments, with the standard deviation for both determinations being about ±14 percent. Analysis of variance of both the cell counting and the enzyme assays indicated that any results differing by more than 30 percent were significantly different ($P < .05$).

The results of the proteolytic assay are shown in Table 1 for cultures exposed to the three anti-inflammatory drugs for 0 to 6 hours with and without the simultaneous addition of inhibitors of protein biosynthesis. These data indicate that strain L fibroblasts contained normally no detectable amounts of proteolytic activities at *p*H 7.5. Significant amounts of this activity were demonstrable within 2 hours after exposure to all three anti-inflammatory drugs, however. Proteolytic activity was maximum after 4 hours. Almost all of this activity was eliminated by the simultaneous addition of either cycloheximide or actinomycin D, the latter agent being slightly more effective than the former.

Separate experiments indicated that adding either cycloheximide or actinomycin D to the induced cell homogenates had no effect on the enzymatic activities of these homogenates; hence neither drug was an inhibitor of proteolytic or collagenolytic activities.

The collagenolytic capacity of these homogenates was also determined (Table 2). Normal strain L fibroblasts contain collagenolytic activity (*p*H 5.5) only after treatment with three anti-inflammatory drugs. This activity was maximum 4 to 5 hours after drug treatment, and almost all of the collagenolytic activity of these cultures was eliminated by simultaneous treatment of the cells with either cycloheximide or actinomycin D. Cells to which no drug was added did not contain any commensurable amounts of either *p*H 7.5 proteolytic or *p*H 5.5 collagenolytic activities, even after 6 hours of incubation.

In other experiments, the cells alone and the medium alone were assayed for collagenolytic and proteolytic activities 4 hours after induction by oxyphenylbutazone or cortisol succinate. More than 50 percent of these activities was found in the medium. Almost all of these activities remaining in the cells could be extracted by gentle washing of the cells in isotonic saline. Thus most of these enzymatic activities could be easily released from the cells into the extracellular fluid. Finally, diploid human fibroblasts (*16*) were grown into confluent monolayers. Homogenates of these cells normally had no collagenolytic or proteolytic (at *p*H 7.5) activities. Within 4 hours after exposure to oxyphenylbutazone (20 $\mu$g/ml) however, they (three different cultures) contained proteolytic activity at *p*H 7.5 equivalent to an average of 60 $\mu$g of tyrosine equivalents per $4 \times 10^6$ cells. They also contained an average collagenolytic activity equivalent to 37 $\mu$g of hydroxyproline per $4 \times 10^6$ cells. These enzymatic activities were reduced to nearly zero by treatment of the cultures with actinomycin D.

Thus both animal and diploid human fibroblasts in confluent monolayers were induced by anti-inflammatory drugs (presumably by the derepression of an operon) to produce and release both collagenolytic and proteolytic activities where no measurable amounts of such enzymatic activities had existed previously in these cells. It is not at all clear what relationship, if any, exists between the anti-inflammatory and enzyme-inducing properties of these drugs.

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